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## Time-dependent regulation of muscle caveolin activation and insulin signalling in response to high-fat diet

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### ABSTRACT

**We studied the effect of high-fat diet on the expression and activation of the three caveolins in rat skeletal muscle and their association with the insulin signalling cascade. Initial response was characterized by increased signalling through Cav-1 and Cav-3 phosphorylation, suggesting that both participate in an initial acute response to the calorie surplus. Afterwards, Cav-1 signalling was slightly reduced, whereas Cav-3 remained active. Late chronic phase signalling through both proteins was impaired inducing a prediabetic state. Summarizing, caveolins seem to mediate a time-dependent regulation of insulin cascade in response to high-fat diet in muscle.**

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### 1. Introduction

Obesity is commonly attributed to an imbalance between food intake and energy expenditure resulting in the storing of energy excess as fat, primarily in adipose tissue and secondly in skeletal muscle, liver, pancreas and brain [1]. Different genes have been related to obesity development, such as the 3 major isoforms of caveolins, Cav-1, Cav-2 and Cav-3 (18–24 kDa), which are the main structural components of caveolae [2]. Cav-1 is most abundantly expressed in terminally differentiated cells, such as adipocytes, fibroblasts, epithelial and endothelial cells. Cav-2 is coexpressed with Cav-1, forming hetero-oligomers that require Cav-1 for proper membrane localization. Finally, Cav-3, whose function is similar to Cav-1, is considered as the muscle-specific member of the caveolin family, although it has also been found in astrocytes and chondrocytes [3,4]. Interestingly, Cav-1 is also expressed in skeletal muscle, where it interacts with Cav-3 in a specific manner [5].

Caveolae have been related to endocytosis of different molecules or particles, such as cholesterol and fatty acids [6], but they act also as scaffolding structures for the organization of a variety of signal transduction proteins [7]. Thus, there is considerable evidence of

caveolin involvement in the modulation of insulin signalling and therefore in the regulation of the intermediary metabolism, which becomes altered in obesity, and associated with metabolic disorders such as insulin resistance and type 2 diabetes [7]. For instance, Cav-3 deficient mice develop late-onset obesity accompanied by insulin resistance in key peripheral tissues and abnormal lipid metabolism [8,9].

Therefore, it is clear that caveolins may play an important regulatory role in muscle insulin signalling. A better understanding of the insulin signalling deregulation that accompanies obesity is a prerequisite to improve therapeutic treatments. Thus, in this work we sought to investigate the effect of high-fat diet feeding in caveolin expression and activation in skeletal muscle and its relation with the deregulation of insulin signalling and the onset of a prediabetic state.

### 2. Materials and methods

#### 2.1. Animals, diets and experimental design

Male Wistar rats (250–300 g), purchased from the Applied Pharmacobiology Center (CIFA, University of Navarra, Spain), were housed under controlled conditions of temperature ( $22 \pm 2^\circ\text{C}$ ), relative humidity ( $55 \pm 10\%$ ) and light cycle (8 a.m. to 8 p.m.). Fifty-six animals were assigned to two different dietary groups for 72 days. The control group ( $n = 28$ ) had ad libitum access to standard

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laboratory pelleted diet (Harlan Iberica, Barcelona, Spain), whereas the second group ( $n = 28$ ) was fed a fat-rich cafeteria diet [10]. Rats were sacrificed by decapitation, after overnight fasting, in four moments during the experimental period: 4, 12, 35 and 72 days. Each group was made up of eight control (C) and eight high-fat fed animals (HF), excepting the groups of day 72 ( $n = 4$ , each). After the sacrifice, trunk blood was collected and gastrocnemius skeletal muscle immediately isolated, frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$ . All the procedures were performed according to national guidelines and under care of the Animal Care and Use Committee at the University of Navarra.

## 2.2. Analysis of blood samples

Serum glucose was analyzed using a Cobas Mira autoanalyzer (Roche Diagnostics, Basel, Switzerland) by enzymatic routine procedures. Serum insulin levels were measured by ELISA (Mercodia AB, Uppsala, Sweden). HOMA index was calculated as fasting insulin ( $\mu\text{U/mL}$ )  $\times$  fasting glucose ( $\text{mmol/L}$ )/22.5.

## 2.3. Reverse transcription (RT)-PCR

Total RNA was extracted from frozen skeletal muscle with Trizol (Invitrogen, Carlsbad, CA, USA), purified by DNase treatment (DNA-free™ kit, Applied Biosystems, Austin, TX, USA) and used as a template to generate first strand cDNA using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Quantitative real-time PCR was performed as described by the provider (Applied Biosystems, Austin, TX, USA) using an ABI-PRISM 7300 HT Sequence Detection System and Taqman probes for rat Cav-1, Cav-2, Cav-3, GLUT-4, IR, IRS-1 and IRS-2. GAPDH was used as internal control for efficiency of RT-PCR and subsequent normalization. The results were calculated by the  $2^{-\Delta\Delta\text{Ct}}$  method [11].

## 2.4. Western blot analyses

For Western blot analysis, skeletal muscle samples were homogenized with lysis buffer. Ten to fifteen micrograms protein were electrophoretically separated and incubated with specific primary antibodies at the suitable dilution and following a standard protocol [12]. The blots were stripped by immersion in Red-Blot Plus solution 10X for 10 min at room temperature (Millipore, Billerica, MA, USA) and reprobed with different antibodies, using the same procedure as described above. Cav-1 (1/50 000), Cav-2 (1/20 000) and Cav-3 (1/10 000) antibodies were from Santa Cruz Biotechnology Inc., CA, USA; Insulin Receptor (1/10 000) and Phosphotyrosine (1/10 000), from Cell Signalling Technology. The secondary antibodies were Anti-Mouse (1/10 000), from Amersham Biosciences-GE Healthcare, WI, USA, Anti-Rabbit (1/10 000), from Sigma Aldrich, St. Louis, MO, USA, and Anti-Goat (1/10 000), from Santa Cruz Biotechnology Inc.

## 2.5. Immunoprecipitation

For immunoprecipitation, samples containing 500  $\mu\text{g}$  of total protein were incubated with antibodies against Cav-1 (1/100 v/w), Cav-3 (1/100 v/w) and IR (1/50 v/v) following the supplier's protocols (Santa Cruz Biotechnology Inc. and Cell Signalling Technology), before being analyzed by Western blot with the corresponding antibodies, as described above, and quantified by densitometry.

## 2.6. Data analysis

The results were expressed as the mean  $\pm$  S.E.M. Data were compared using Mann–Whitney  $U$  test, while correlation analyses

were performed using Spearman's  $\rho$ . All analyses were performed using SPSS version 15.0 for Windows.

# 3. Results

## 3.1. Body weight and blood determinations

Rats consuming the high-fat diet (HF) gained significantly more weight ( $P < 0.05$ ) than those receiving the control diet, increasing body fat percentage and decreasing relative muscle weight with respect to total body weight since day 12 (Fig. 1). They also developed hyperglycemia, hiperinsulinemia and insulin resistance at the end of the experimental period (Fig. 1).

## 3.2. mRNA and protein levels of caveolins

Cav-3 mRNA, measured by RT-PCR, resulted significantly increased in HF-fed rats from day 12 as compared to the control group (Fig. 2). Cav-1 and Cav-2 show a similar expression pattern than Cav-3, although the differences were not significant (Fig. 2).

Caveolin protein contents in skeletal muscle, quantified by Western blot analysis, were not affected by the diet (Fig. 3A). However, Cav-1 and Cav-3 show a positive and Cav-2 an inverse correlation to body weight (Fig. 3B).

## 3.3. Protein phosphorylation of Cav-1, Cav-3 and IR

Phosphorylation levels of IR, Cav-1 and Cav-3 were increased in HF-fed rats at day 4, as a sort of initial response (Fig. 4), more evident for IR ( $P < 0.05$ ) and Cav-3 ( $P = 0.053$ ). However, from day 12 ahead, IR phosphorylation level in HF animals was the same as in control fed rats (Fig. 4), indicating that there is no response of IR to the increased insulin circulating level.

## 3.4. mRNA expression of insulin pathway proteins

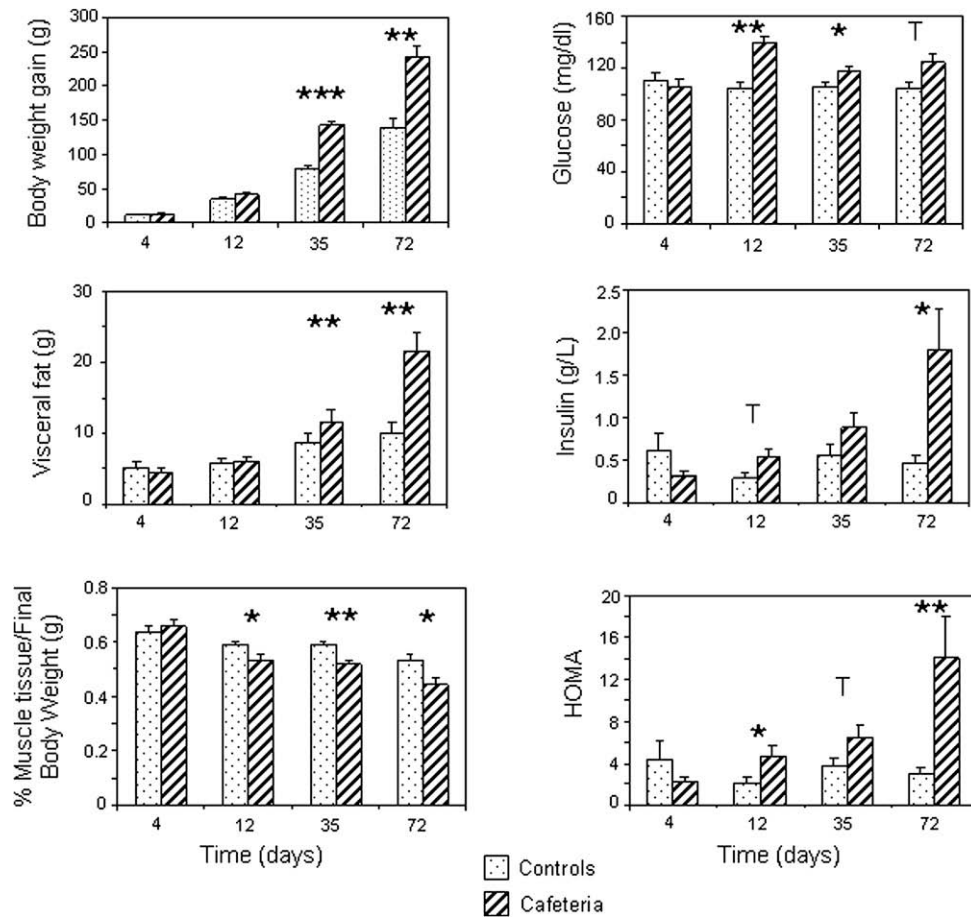
Neither IR nor GLUT4 mRNAs were modified by the diet (Fig. 5). IRS-1 increased its expression in HF animals at day 4, as an early response to the diet, but from day 12 ahead, it gradually decreased ( $P < 0.05$  at day 72). On the other hand, IRS-2 showed an inverse expression pattern to IRS-1, being inhibited up to day 35 but significantly increased at day 72 (Fig. 5).

# 4. Discussion

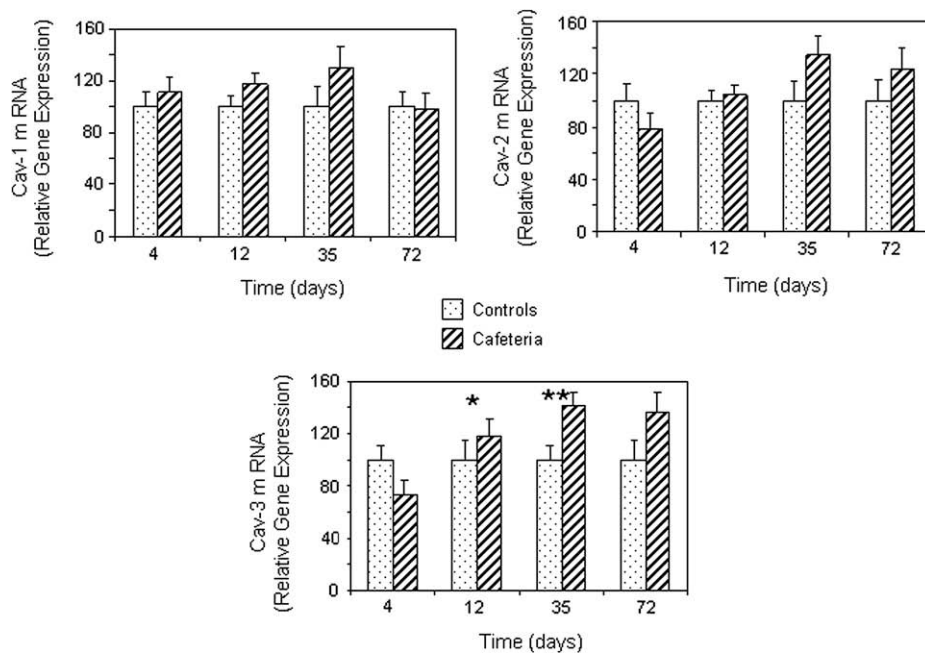
After 10 weeks of high-fat cafeteria diet, rats developed a prediabetic state characterized by hyperinsulinemia and insulin resistance, as previously reported [13]. Skeletal muscle is the major organ responsible for glucose uptake under insulin-stimulated conditions [14], and is generally considered the most important site for insulin resistance [15]. An impairment of the initial steps of insulin signalling transduction pathways could contribute to the defect in insulin-stimulated glucose uptake in skeletal muscle, thus resulting in insulin resistance. In fact, two mechanisms have been described in relation to lipid-induced muscle insulin resistance: acute free fatty acid elevation, and prolonged muscle lipid accumulation [16].

In this study, increased insulin resistance in animals fed a fat-rich diet is evidenced by reduced insulin receptor (IR) phosphorylation since day 12 and IRS-1 mRNA at day 72, confirming data from other groups [17]. Cav-1 is also downregulated since day 12, suggesting that this protein may be involved in the malfunction of the classical insulin pathway that could result in insulin resistance [18].

It is known that caveolin subtypes 1 and 3 play an important role in maintaining insulin signalling and cellular energy metabo-



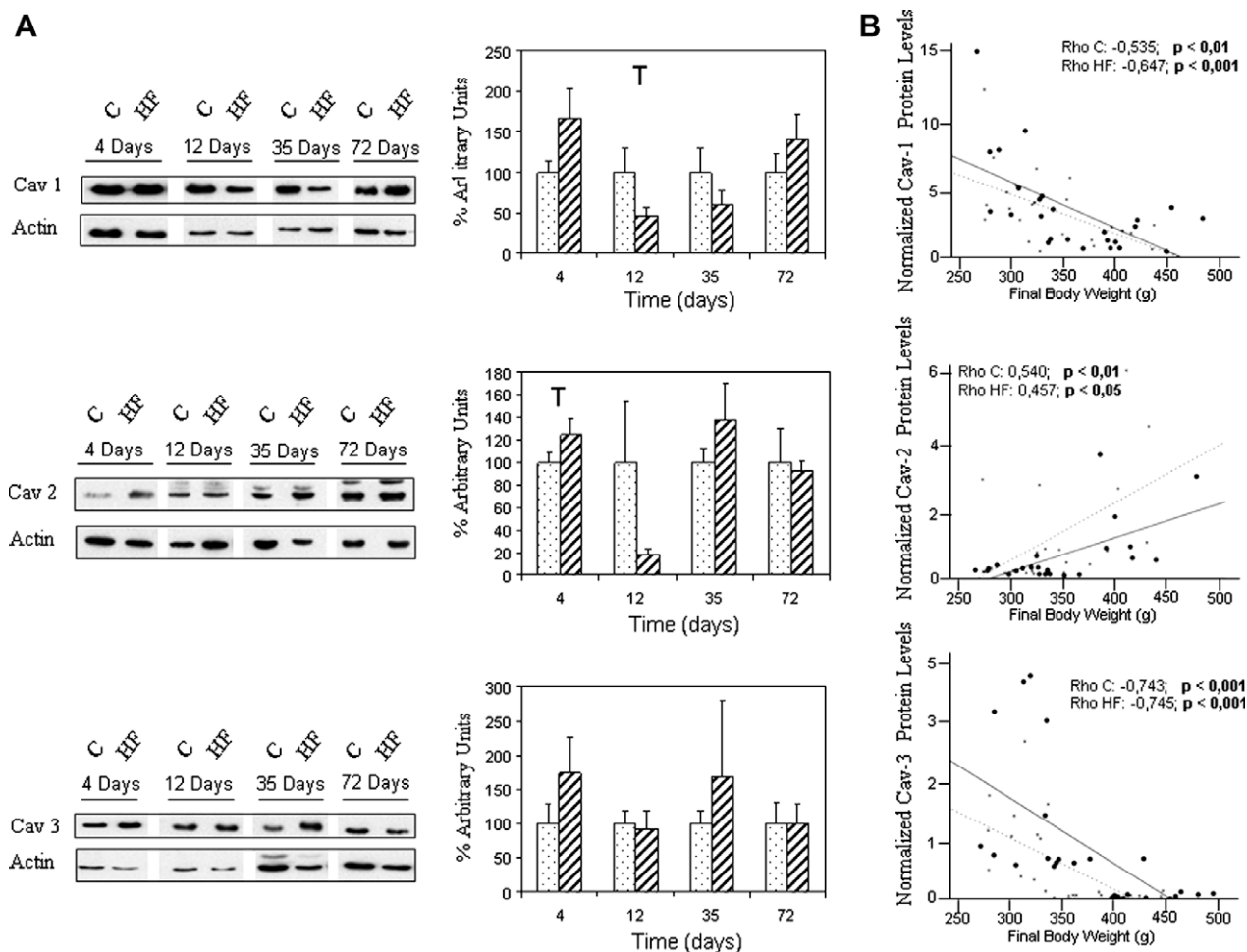
**Fig. 1.** Effect of high-fat diet in body weight and serum measurements (mean  $\pm$  S.E.M.). C vs. HF, \*\*\*P < 0.001; \*\*P < 0.01; \*P < 0.05; T < 0.10.



**Fig. 2.** Effect of high-fat diet in caveolin mRNA levels (mean  $\pm$  S.E.M. of the ratio between each caveolin and GAPDH expression, and referred to control group gene expression (C = 100)). C vs. HF, \*\*P < 0.01; \*P < 0.05.

lism [5]. Thus, Cav-1 knockout mice, when fed a HF-fed diet, show resistance to diet-induced obesity, dyslipidemia and important fat tissue pathology [19,20], which has been attributed to defective

insulin signalling [20]. In adipocytes, a dramatic reduction in IR phosphorylation has been described [20], which has been explained by the reduction of the IR protein itself with normal mRNA



**Fig. 3.** (A) Effect of high-fat diet in caveolin protein levels in skeletal muscle (mean  $\pm$  S.E.M. of the ratio between each caveolin and actin and are referred to control group gene expression (C = 100)). C vs. HF,  $T < 0.10$ . (B) Association analysis between muscle caveolin protein levels and final body weight ( $R$  = Spearman's  $\rho$ ): C (black line and spots) and HF (dotted line and x marks). Protein levels are expressed as the normalized ratio between caveolin and actin signals for each individual sample.

levels. However, the amount of IR protein in other tissues, such as skeletal muscle, remained unchanged.

Cav-3 knockout mice show loss of caveolae in skeletal muscle plasma membrane and abnormalities in the organization of the T-tubule system [21], developing adiposity, dyslipidemia and hyperglycemia [9]. In skeletal muscle, the protein expression of IR and other molecules involved in insulin signalling are unaltered, whereas phosphorylation of IR, IRS or Akt/PKB are all attenuated and insulin-stimulated glucose uptake is decreased [8].

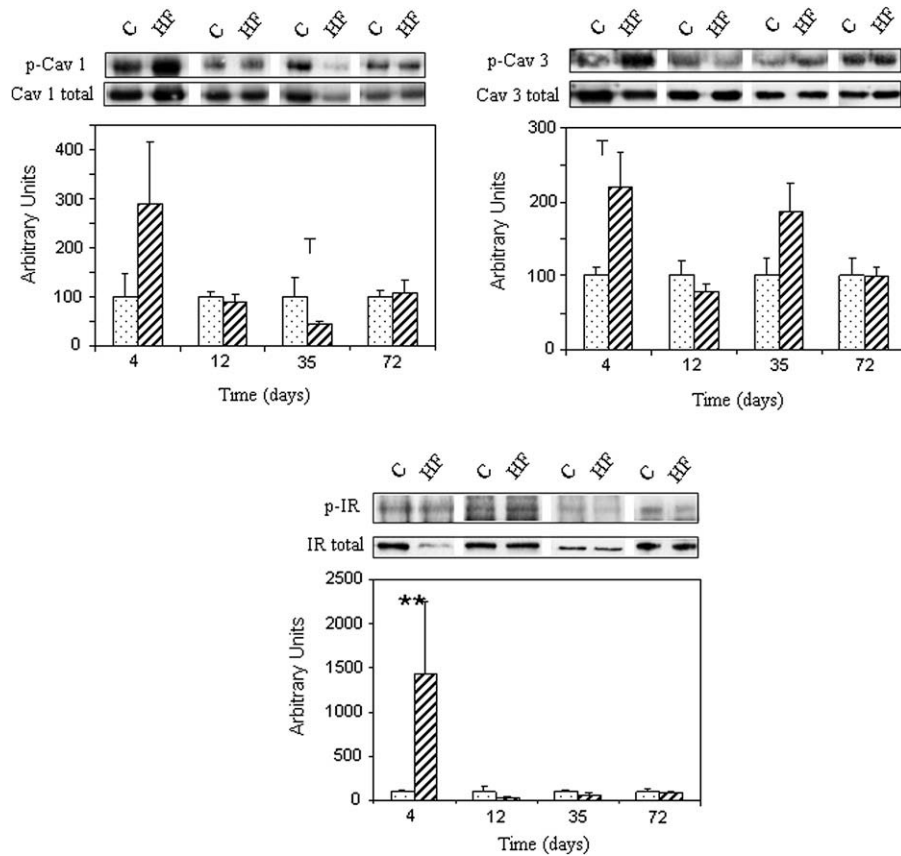
Our results indicate that HF diet seems to induce, in muscle insulin signalling, a time dependent response with three different stages. In the initial response against the excess of nutrient input (day 4), IR, Cav-1 and Cav-3 are highly phosphorylated and IRS-1 mRNA upregulated. Cav-1 is also slightly upregulated, as reported by other authors using similar diets [22]. This early response of Cav-1 could be related to the high amount of cholesterol and the overload of fatty acids in the diet [22], but also to the release of the inflammatory cytokine TNF- $\alpha$  [23]. Therefore, we suggest that, in this prompt response, insulin-related pathways might be activated by a coordinated mechanism mediated by both caveolins and IR, in an attempt to effectively fight against the excess of dietary nutrients in order to maintain glucose balance.

After 12 days, the initial response change towards an adaptation to the new metabolic state, probably mediated by damage in the main insulin pathway and the overwhelming of the cell with more fat and glucose that it can oxidize. Subsequently, at day 35, a de-

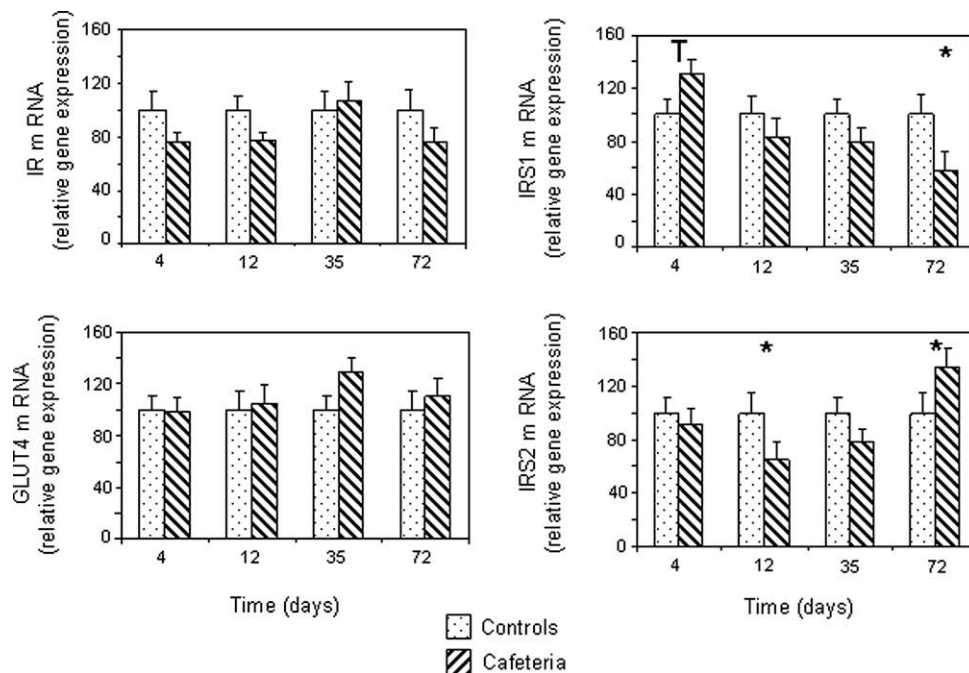
layed response characterized by the establishment of an obese metabolism was observed [24], with the animals showing hyperglycemia, hyperinsulinemia and insulin resistance. In accordance to this, Cav-1 and IR are no longer activated. Hyperglycemia could also be contributing to Cav-1 downregulation at this stage, as other authors have shown in Schwann cells [25]. Cav-1 expression pattern is similar to that observed in adipose tissue in previous studies, while Cav-2 behaves in the opposite way [26,27], suggesting that they are inversely regulated. In adipocytes Cav-1 co-localizes with IR and positively regulates the activation of IR upon insulin stimulation [28], but the role of Cav-1 in muscle insulin signalling has received little attention, probably because Cav-3 is the muscle-specific isoform. Our results suggest that insulin signalling could be downregulated due to a decrease in Cav-1 and IR phosphorylation, in accordance to other authors that have reported reduced muscle insulin-stimulated glucose uptake after siRNA downregulation of Cav-1 [5].

However, we also find that Cav-3 expression and phosphorylation are still upregulated at day 35, suggesting that Cav-3 may be actually playing a role in the regulation of insulin signalling in this stage. It has been described that, although IR phosphorylation in skeletal muscle is not affected by Cav-3 downregulation, Cav-3 is essential in the maintenance of interactions with the cell cytoskeleton through other caveolae molecules, such as flotillin-1 [29]. Thus, insulin stimulation in skeletal muscle cells results in the translocation to the plasma membrane of internal domains





**Fig. 4.** Effect of high-fat diet in Cav-1, Cav-3 and IR phosphorylation in skeletal muscle (mean  $\pm$  S.E.M. of the ratio between each phosphoprotein and total caveolin and are referred to control group gene expression (C = 100)). C vs. HF, \*\* $P < 0.01$ ; T < 0.10.



**Fig. 5.** Effect of high-fat diet in mRNA levels of IR, IRS1, IRS2 and GLUT4 in skeletal muscle (mean  $\pm$  S.E.M. of the ratio between each gene and GAPDH expression and are referred to control group gene expression (C = 100)). C vs. HF, \* $P < 0.05$ ; T < 0.10.

containing signalling pathway intermediates and GLUT4, in a process that is facilitated by Cav-3–flotillin-1 interaction [29]. In this context, in adipocytes, a dominant Cav-3 mutant inhibits insulin-

stimulated GLUT4 translocation and activation of TC10 (a Rho family GTPase), in a manner independent from PI-3 kinase pathway [30].

Therefore, we propose that, in a second stage, the continuous high-fat diet exposure decreases Cav-1 phosphorylation and IR activation, resulting in a reduction of GLUT-4 translocation by similar mechanisms as in adipocytes as suggested by the decrease in IRS-1 mRNA levels. However, cafeteria diet also induces Cav-3 activation, which through cytoskeletal modifications could regulate spatial and temporal compartmentalization of GLUT4 vesicles, in an alternative attempt to maintain glucose homeostasis.

Finally, in a late chronic phase (day 72), Cav-3 is also dephosphorylated indicating that both signalling pathways are impaired. This will cause strong insulin resistance, marked hyperglycemia and the establishment of a prediabetic state. This hypothesis is supported by other authors, who observed reduced levels of Cav-3 in cardiomyoblasts in insulin resistant state [31]. Interestingly IRS-2 mRNA levels are significantly higher at this stage, but how this increase might be linked to the metabolic situation is something that remains to be tested.

In summary, we suggest a coordinated mechanism involving caveolins and IR in muscle cells, in which feeding a high-fat cafeteria diet appears to induce a three stage time-dependent insulin response: initial, delayed and chronic. The initial response would be characterized by an intense signalling through Cav-1 and Cav-3 phosphorylation. Afterwards, the intensity of signalling would be reduced since Cav-1 is inhibited but Cav-3 remains active. Finally, in the late phase, signalling through both proteins would be impaired inducing a prediabetic state.

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